AGRICULTURAL MATERIALS

Determination of Phytase Activity in Feed by a Colorimetric Enzymatic Method: Collaborative Interlaboratory Study

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Fourteen laboratories participated in a collaborative study (coded fyt9404) and 13 laboratories participated in a study (coded fyt9410) to validate a colorimetric assay for determination of microbial phytase activity in feed. For each study, all laboratories received 6 laboratory samples provided by one commercial supplier (phytase activity levels within the range of 200–400 per kg) to be analyzed in duplicate. Method performance was calculated and statistical calculations were executed according to AOAC guidelines. Results from 3 laboratories for study fyt9404 and from one laboratory for study fyt9410 were excluded from statistical analysis because of invalid data determined during initial review by Youden pair, value versus laboratory. For study fyt9404, repeatability relative standard deviation (RSD_r) values ranged from 6.2 to 8.6%, and reproducibility relative standard deviation (RSD_R) values ranged from 14.1 to 27.6%. No outliers were identified. For study fyt9410, RSD_r values ranged from 3.9 to 7.9%, and RSD_R values ranged from 14.0 to 20.5%. With outliers excluded, RSD_r values ranged from 2.5 to 7.9%, and RSD_R values ranged from 14.0 to 20.5%.

Phytase has been used successfully as a feed additive for poultry and pigs (monogastric animals) to improve phosphorus availability in feed. The enzyme is found in various sources (e.g., seeds), and many microorganisms produce phytase (e.g., *Aspergillus* molds). For application of phytase in the feed industry, a reliable and easy colorimetric enzymatic method was developed for determination of phytase activity to allow comparison of enzyme preparations based on the same unit definition and the same method.

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The recommendation was approved by the Methods Committee on Feeds, Fertilizers, and Related Agricultural Products and was adopted by the Official Methods Board of AOAC INTERNATIONAL. *See* "Official Methods Board Actions," (1999) *Inside Laboratory Management*, November/December issue.
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Collaborative Study

Fourteen and 13 laboratories participated in 2 separate collaborative studies coded fyt9404 and fyt9410, respectively. These studies were executed according to the guidelines described by Youden (1) and ISO 5725 (2, 3). Each laboratory received the procedure and 6 test samples per study. Phytase activity level of samples was within the range of 200–400 phytase units (FTU)/kg. The laboratory samples included feeds for layers, layer breeders, broilers, turkeys, pigs, and piglets. The samples were shipped by commercial carrier. Collaborators were instructed to refrigerate (4 ± 2°C) the laboratory samples during storage. Each test sample was analyzed in duplicate on the same day. Collaborators were asked to prepare test solutions containing 0.01–0.07 FTU/2 mL.

AOAC Official Method 2000.12
Phytase Activity in Feed
Colorimetric Enzymatic Method
First Action 2000

(Applicable to the determination of phytase activity in animal feed in the range of 200–400 phytase units [FTU]/kg.)

Caution: See Appendix B, "Enzyme Preparations."

See Table **2000.12A** for the results of the interlaboratory study supporting acceptance of the method.

A. Principle

Phytase is incubated with sodium phytate, liberating inorganic phosphate at 37 ± 0.1°C and pH 5.5. Incubation is stopped by adding acid molybdate–vanadate reagent which also produces a colored complex with the phosphate produced. The color of the yellow vanadomolybdophosphor complex, which is a measure for the amount of phosphate, is measured at 415 nm.

B. Apparatus

(**a**) *Water bath*.—Thermostatically controlled to 37.0 ± 0.1°C with circulating water.

(**b**) *Diluter*.—Provided with 0.500 and 5.000 mL cylinders, Hamilton Microlab 1000 (Hamilton Bonaduz AG; Bonaduz, Switzerland), or equivalent.

(**c**) *Dispensers*.—Adjusted to 4.00, 50.0, and 100 mL.

Table 2000.12A. Interlaboratory study results for the determination of phytase activity in feed using a colorimetric enzymatic method

Feed[a]	FTU/kg	No. labs [b(c)]	r	s_r	% RSD_r	R	s_R	% RSD_R
Piglet	269	10 (1)	48.2	17.2	43.2	325.4	116.2	6.4
Layer	367	10 (1)	66.4	23.7	33.6	345.0	123.2	6.5
Fattening pig	299	10 (1)	72.2	25.8	27.6	231.6	82.7	8.6
Broiler	317	8 (0)	70.0	25.0	20.5	182.0	65.0	7.9
Turkey	346	8 (0)	53.5	19.1	22.4	216.4	77.3	5.5
Layer breeders	208	8 (0)	33.9	12.1	27.6	160.7	57.4	5.8

[a] Blind duplicate test sample pairs.

[b(c)] Where, b = number of laboratories retained after eliminating outliers; (c) = number of laboratoriess removed as outliers.

(**d**) *Spectrophotometer*.—Operating at 415 nm, with a spectral bandwidth 8 nm, with 10.00 mm continuous-flow cuvette with debubbler system, Pye Unicam PU 8600 (Helma GmbH & Co. KG, Postbox 1163, D-79371, Müllheim, Germany), or equivalent.

(**e**) *Centrifuge*.—Relative centrifugal force of 3000 × *g*, provided with rotor with inserts for 11 centrifuge tubes of 15 mL each.

(**f**) *Vortex mixer*.—2500 rpm.

(**g**) *Laboratory mill*.—Provided with 1 mm sieve and 6-tooth rotor, Ultra centrifugal mill ZM 100 (Retsch GmbH & Co. KG, Postbox 1554, D42759 Haan, Germany), or equivalent.

(**h**) *Filter*.—Paper filters, grade 595, 0.16 mm (Schleicher & Schuell; Dassel, Germany), or equivalent.

C. Reagents

Note: Prepare all solutions containing phytase in glassware. Plastic (disposable) material interferes with the assay. Do not use phosphoric acid or detergents that contain phosphate to wash glassware.

Use ultra high purity water, 18 MW-cm resistivity, for the preparation of all reagents and test solutions.

(**a**) *Phytase enzyme*.—A highly concentrated phytase enzyme preparation can be obtained from DSM Food Specialties, R&D Analysis, 010-0585, PO Box 1, 2600 MA Delft, The Netherlands, or equivalent. Use activity value certified by manufacturer.

(**b**) *Tween 20 solution*.—Quantitatively transfer 10.0 g Tween 20 (polyoxyethylene sorbitan monolaureate, for synthesis grade; Merck KgaA, Postbox 64271 Darmstadt, Germany) into 100 mL volumetric flask, dissolve in 80 mL H_2O, dilute to volume with H_2O, and mix well. Prepare fresh before use.

(**c**) *Dilution buffer*.—Transfer 1.76 g 4M acetic acid (24 mL diluted to 100 mL), 30.0 g Na acetate·$3H_2O$, and 0.147 g $CaCl_2 \cdot 2H_2O$ into 900 mL H_2O in 1 L volumetric flask. Adjust to pH 5.5 by dropwise addition of acetic acid. Add 1 mL Tween 20 solution, (**b**), and dilute to volume with H_2O. Prepare fresh before use.

(**d**) *Feed buffer*.—Transfer 30.0 g Na acetate·$3H_2O$ and 10.05 g $CaCl_2 \cdot 2H_2O$ into 1 L volumetric flask, dissolve in 900 mL H_2O, and add 0.1 g Tween 20. Adjust to pH 5.5 by dropwise addition of acetic acid and dilute to volume with H_2O. Prepare fresh before use.

(**e**) *Phytic acid*.—Dodecasodium salt, from rice (P3168, Sigma Chemical Co., PO Box 14508, St. Louis, MO 63178, USA, or equivalent).

(**f**) *Phytic acid substrate*.—Quantitatively transfer 8.40 g phytic acid, (**e**), into 1 L volumetric flask and dissolve in 900 mL dilution buffer, (**c**). Adjust to pH 5.5 by dropwise addition of 4M acetic acid (24 mL diluted to 100 mL), and dilute to volume with H_2O. Prepare fresh before use. If other than $\cdot 12H_2O$ product supplied, adjust weight accordingly.

(**g**) *Ammonium heptamolybdate stock solution*.—Transfer 100 g ammonium heptamolybdate ($H_{24}Mo_7N_6O_{24} \cdot 4H_2O$) into 1 L volumetric flask and dissolve in ca 900 mL H_2O. Add

Table 2000.12B. Serial dilutions for calibration line solutions

Stock solution	Stock solution, mL	Dilution buffer, **C**(**c**), to be added, mL	Diluted calibration line solution, mL	Dilution buffer, **C**(**c**), to be added, mL	Solution to be incubated, mL	Dilution buffer, **C**(**c**), to be added, mL	Activity
A	0.250	4.750	0.080	9.920	0.100	1.900	0.007
B	0.250	4.750	0.150	9.850	0.100	1.900	0.014
A	0.250	4.750	0.100	4.900	0.200	1.800	0.036
B	0.250	4.750	0.150	4.850	0.200	1.800	0.054
A	0.250	4.750	0.200	4.800	0.200	1.800	0.072

10 mL NH_4OH and dilute to volume with H_2O. Solution is stable up to 1 month when stored at ambient temperature (20–25°C) shielded from light.

(**h**) *Ammonium vanadate stock solution*.—Transfer 2.35 g ammonium vanadate (NH_4VO_3) into 1 L volumetric flask and completely dissolve in ca 400 mL H_2O at 60°C. Add slowly while swirling 20 mL HNO_3 (7 + 13), cool to room temperature, and dilute to volume with H_2O. Solution is stable up to 1 month when stored at ambient temperature (20–25°C) shielded from light.

(**i**) *Color stop mixture*.—Mix 250 mL heptamolybdate solution and 250 mL vanadate solution and add slowly, while swirling, 165 mL HNO_3 (7 + 13). Cool to room temperature, and dilute to 1 L with H_2O. Prepare fresh before use.

D. Determination

Note: Some metal ions, such as Cu, Fe, Zn, etc., may inhibit the enzyme reaction when present in millimolar range.

(**a**) *Test sample pretreatment*.—(*1*) *Feeds*.—Grind ca 100–150 g laboratory sample with mill, **B**(**g**). Grind until all material has passed through 1 mm sieve. Mix test sample thoroughly and in 125 mL glass beakers, weigh, in duplicate, ca 5 g test portion to the nearest 1 mg. Add 50 mL feed buffer, **C**(**d**), and stir with magnetic stirrer 60 min. Filter the top layer through paper. Dilute filtered solution with dilution buffer, **C**(**c**), to phytase activity within the range of 0.01–0.07 FTU/2 mL solution, and store in melting ice until incubation. Perform final dilution of test portion and in duplicate (test sample and test sample blank).

(*2*) *Preparation of calibration line solutions*.—Before use, let phytase standard, **C**(**a**), attain ambient temperature (20–25°C). In duplicate, in 200 mL volumetric flasks (stock solutions A and B), weigh to nearest 1 mg amounts of phytase standard, corresponding to 36 000 FTU. Dissolve in dilution buffer, bring to volume with dilution buffer, **C**(**c**), and mix. Perform the following serial dilutions of phytase standard solutions A and B. Prepare final dilutions in duplicate (standard and standard blank). *See* Table **2000.12B**.

Prepare fresh phytase standard solutions and dilute calibration line solutions before use. Store calibration line solutions in melting ice until incubation and analyze as described below.

(**b**) *Incubation*.—(*1*) *Calibration line solutions and test portions*.—Monitor time exactly using a stopwatch. Starting at time = 0 min, in the order of the series and at regular time intervals, place one of the tubes to be incubated into water bath

Table 1. Collaborative study results of phytase activity (FTU/kg) in feed enzyme preparations: study fyt9404[a]

	Samples[b]					
	Piglet feed		Layer feed		Fattening pig feed	
Lab	A	B	A	B	A	B
01	158	196	344	325	226	241
02	460[c]	510[c]	670[c]	630[c]	450	400
03	200	180	310	340	250	240
04	200	190	310	300	200	220
05	205	213	336	353	230	241
08	257	241	403	422	251	248
10	910[c]	980[c]	810[c]	890[c]	690[c]	640[c]
11	255	246	426	423	346	357
13	220	210	260	270	360	340
14	440[c]	460[c]	200	280	440	350
Mean	212		331		299	
s_r	13.2		23.1		25.8	
s_R	30.0		66.3		82.7	
RSD_r, %	6.2		7.0		8.6	
RSD_R, %	14.1		20.0		27.6	
r[d]	37.0		64.7		72.2	
R[e]	84.0		185.6		231.6	

[a] Method performance after outliers were excluded.

[b] Blind duplicates, mean activity of duplicate analysis for each sample.

[c] Invalid data.

[d] $r = 2.8 \times s_r$.

[e] $R = 2.8 \times s_R$.

Table 2. Collaborative study results of phytase activity (FTU/kg) in feed enzyme preparations: study fyt9410[a]

	Samples[b]					
	Broiler feed		Turkey feed		Layer breeders feed	
Lab	A	B	A	B	A	B
01	287	295	321	324	202	204
03	405	334	402	358	191[c]	235[c]
04	320	320	360	380	230	220
05	298	288	324	318	187	192
07	341	312	363	386	231	246
08	303	295	333	338	198	194
11	427	422	467	466	300	299
13	241	179	170[d]	224[d]	94[d]	99[d]
Mean	317		367		225	
s_r	25.0		14.5		5.6	
s_R	65.0		51.4		41.0	
RSD_r, %	7.9		3.9		2.5	
RSD_R, %	20.5		14.0		18.2	
r[e]	70.0		40.6		15.7	
R[f]	182.0		143.9		114.8	

[a] Method performance calculated after outliers were excluded.
[b] Blind duplicates, mean activity of duplicate analysis for each sample.
[c] Rejected by Cochran's test.
[d] Invalid data.
[e] $r = 2.8 \times s_r$.
[f] $R = 2.8 \times s_R$.

at 37.0 ± 0.1°C. Starting at time = 5.0 min, in the same order of the series and at the same regular time intervals, add 4.00 mL phytic acid substrate, **C(f)**, at 37.0 ± 0.1°C with a dispenser and mix with vortex mixer, **B(f)**. Place tubes into water bath maintained at 37.0 ± 0.1°C. At time = 65.0 min, in the same order and with the same time intervals, terminate incubation by adding 4.00 mL color stop mixture, **C(i)**, with a dispenser, and mix.

(*2*) *Blanks*.—Starting at time = 0 min (stopwatch), in the order of the series and with regular time intervals, place tubes containing blank in water bath maintained at 37.0 ± 0.1°C to equilibrate. At time = 5.0 min, in the same order of the series and with the same time intervals, add 4.00 mL color stop mixture, **C(i)**, with a dispenser, mix, and place tubes on work bench. Next, add 4.00 mL phytic acid substrate, **C(f)**, to all blank tubes, and mix.

Centrifuge the calibration line, test portion, and blank tubes for 5 min at 3000 × *g*. Measure absorbance at 415 nm with spectrophotometer, zeroing the instrument with H_2O. Repeat the analysis with a more diluted solution whenever the activity found is > 0.07 FTU/2 mL solution to be incubated or if absorbance of incubated solution is >1.000.

E. Expression of Phytase Activity

One phytase unit is defined as that quantity of enzyme that will liberate 1 mol inorganic *ortho*-phosphate per minute under the conditions of the assay.

Calculation of enzyme activity.—Calculate results with a calculator or computer program that can perform parabolic regression. If a calculator or computer program is not available, then calculate enzyme activity manually as follows:

(*1*) Calculate absorbance (A) of calibration line solutions and test sample solutions (A = absorbance test sample – absorbance blank).

(*2*) On linear graph paper, plot exactly calculated activities of calibration line solutions (FTU/2 mL) against corresponding absorbances.

(*3*) Draw best fitting polynomial curve not forcing it through the origin (each calibration line point should deviate no more than 5% from the curve). From this curve, read the activity of test sample solutions in FTU/2 mL.

(*4*) Calculate phytase activity of test samples as follows:

$$\text{Phytase activity, FTU/kg} = (\text{FTU/2 mL to be analyzed}) \times (f)/W$$

where f = total dilution factor of test sample based on test portion weight of 1.000 g; W = weight of test portion, g.

Report final activity of enzyme preparation as average of duplicate analysis to 2 significant figures.

Ref.: *J. AOAC Int.* **84**, 629–633(2001)

Results

Data from 10 of 14 collaborators for study fyt9404 are presented in Table 1. Results from 3 laboratories were not received. One laboratory used another method and was excluded from statistical analysis. Consistently outlying values were observed for 3 laboratories that were excluded from the statistical analysis because of invalid data. There were no indications that this was the result of departures from the protocol. No collaborative comments were received. Repeatability relative standard deviations (RSD_r) for all of the reported data ranged from 6.2 to 8.6%. The Cochran test and the single Grubbs test identified no outliers. Reproducibility relative standard deviation (RSD_R) for all the reported data ranged from 14.1 to 27.6%.

Data from 8 of 14 collaborators for study fyt9410 are presented in Table 2. Results from 4 laboratories were not received. One laboratory used another method and was excluded from statistical analysis. Consistently outlying values were observed for one laboratory that was excluded from the statistical analysis because of invalid data. There were no indications that this was the result of departures from the protocol. No collaborative comments were received. The RSD_r for all of the reported data ranged from 3.9 to 7.9%, and decreased from 2.5 to 7.9% after outliers identified by the Cochran test were excluded. No outliers were identified by the single Grubbs test. The RSD_R for all of the reported data ranged from 14.0 to 20.5%, and did not decrease after outliers were rejected.

Discussion

The purpose of this collaborative study was to evaluate an easy, reliable, accurate method for determination of phytase activity in feed enzyme preparations. The method allows comparison of industrial enzyme preparations in the laboratory and does not represent actual performance of the preparations. The capability of phytic acid to act as complexing agent for removal of traces of metal ions and the presence of protecting protein content in the feed itself are sufficient to eliminate the negative effect of metal ion interference. The precision parameters reported in this study are as expected for such a diverse undertaking and the feed matrix examined. In conclusion, the method has proved to be reliable and accurate.

Recommendation

Based on the results of this study, it is recommended that the colorimetric method for determination of phytase activity in feed enzyme preparations be adopted First Action.

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References

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